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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. §1.53(b)(2)

Atty. Docket: FISCHER16

INVENTOR(S)/APPLICANT(S)			
LAST NAME	FIRST NAME	MI	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY
FISCHER	Bilha		Shoham, Israel
NAHUM	Victoria		Rishon Lezion, Israel

[ ] Additional inventors are being named on separately numbered sheets attached hereto

TITLE OF THE INVENTION (280 characters max)

INORGANIC BORANOPHOSPHATE SALTS

CORRESPONDENCE ADDRESS

Direct all correspondence to the address associated with **Customer Number 001444**, which is presently:

BROWDY AND NEIMARK, P.L.L.C.
624 Ninth Street, N.W., Suite 300
Washington, D.C. 20001-5303

ENCLOSED APPLICATION PARTS (check all that apply)

[X] Specification Number of Pages 40 [X] Applicant claims small entity status. See 37 C.F.R. §1.27

[ ]Drawing(s) Number of Sheets [ ] Other (specify) _____

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The invention was made by an agency of the United Stated Government or under a contract with an agency of the United States Government.

[X] No [ ] Yes, the name of the U.S. Government agency and the Government contract number are:

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Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.

By: _____
Sheridan Neimark
Registration No.: 20,520

Date: February 2, 2004

SN:jlu

BARIL-003 US PROV

INORGANIC BORANOPHOSPHATE SALTS

Inventors: **Bilha FISCHER, Victoria NAHUM**

FIELD OF THE INVENTION

The present invention relates to inorganic boranophosphate salts, that are phosphate mimic, and to their preparation and uses.

BACKGOUND OF THE INVENTION

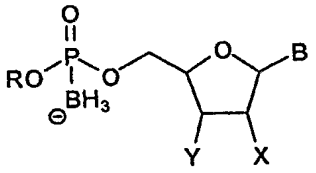
The quest for phosphate bioisosters over the last decades included phosphonates, α-halo-phosphonates, phosphoramidates, and phosphorothioates.

Phosphates and phosphate-containing molecules play a major role in numerous biological systems.[1] The unwanted lability of the ester P-O bond promoted the search for suitable bioisosters, phosphate analogues, which will retain biological activity, but possess diminished lability. The search for bioisosters was initiated by the need to produce phosphate probes for various studies, such as probing stereochemical requirements of enzymes.[2] In addition, phosphate bioisosters were developed for improving the pharmacological effects of nucleotide based drugs, e.g. anti-sense agents.[3]

A widely used isoster of phosphate is phosphorothioate and its analogues, proposed in the pioneering work of Eckstein *et al.* [4] In these analogues the non-bridging oxygen is substituted by sulfur. Other chemical modifications of the phosphate moiety include the

substitution of the labile phosphate ester oxygen by carbon or nitrogen, to give phosphonates and phosphoramidate analogues, respectively.[5]

During the last decade, pioneering studies by Spielvogel and Ramsay-Shaw proposed boranophosphate analogues **1** as bioisosters of natural nucleotides[6] and as important tools for biochemists.[7]



B = A, C, T, G, U
X = H, OH

1a. Y = OH; R = H
1b. Y = OH; R = $P_2O_6^{3-}$
1c. Y = OH; R = PO_3^{2-}
1d. Y = OH; R = 3'-nucleoside
1e. Y = OH; R = 3'-oligonucleotide
1f. R = 3'-ribose

Spielvogel and Ramsay-Shaw proposed in 1990 nucleoside boranophosphate analogues as bioisosters of natural nucleotides. This new class of boron modified nucleotides, that mimic phosphodiesters, phosphorothioates, and methyl phosphonates, was designed for use as potential therapeutic and diagnostic agents. These nucleoside boranophosphates, or borane phosphonates, have a borane moiety (BH_3) in replacement of one of the nonbridging oxygens in the phosphate diester moiety. The BH_3 group maintains the negative charge of a phosphate, but it does not form classical H-bonds and does not coordinate with metal ions. This modification imparts unique characteristics to boranophosphate nucleotides and nucleic acids. The boranophosphate can be considered as a "hybrid" of three well-studied types of modified phosphates, namely, normal phosphate, phosphorothioate, and non-ionic methylphosphonate. The BH_3 group in the

boranophosphates is isoelectronic with oxygen (O) in the normal phosphates, and isolobal (pseudo-isoelectronic) with sulfur (S) in phosphorothioates. The BH_3 group is isosteric with the CH_3 group in the methylphosphonates. Boranophosphates would be expected to share a number of chemical and biochemical properties with phosphorothioate and methylphosphonate analogs.

This emerging field of novel nucleotide bioisosters has expanded significantly and provided many important applications of the boranophosphate analogues. For instance, non-terminal P-boronated nucleotides, existing as a pair of diastereoisomers, were used as stereochemical probes to elucidate enzymatic catalysis.[8] Oligodeoxyribonucleotides bearing boranophosphate linkages have been used for polymerase chain reaction (PCR) sequencing and DNA diagnostics.[9] Boranophosphate nucleotides were found as highly potent and stable P2Y-receptor agonists.[10] Oligonucleotides bearing boranophosphate linkages were regarded as potentially useful anti-sense agents.[11] These analogues were also considered for the treatment of cancer as carriers of ^{10}B isotope in boron neutron capture therapy.[12]

SUMMARY OF THE INVENTION

The present invention relates to salts of the inorganic boranophosphate of the general formula **2**, herein designated Bpi salts, wherein M is a counterion.

In one embodiment, the counterion M is ammonium or it is an inorganic cation derived from an alkali, alkaline earth or transition metal such as but not limited to NH_4^+, Na^+, K^+, Li^+, Ca^{++}, Mg^{++}, Ni^{++}, Cu^{++}, Fe^{++}, Fe^{+++}, Co^{++}, Zn^{++}, Pd^{++}, and Ag^+.

In another embodiment, the counterion M is an organic cation derived from an amine of the formula R_3NH^+, wherein R is C1-C18, preferably C1-C6, alkyl, more preferably ethyl, propyl or butyl, or two of the Rs together with the nitrogen atom to which they are attached form a 3-7 membered ring optionally containing a further heteroatom selected from the group consisting of N, S and O, such as for example pyrrolydine, piperidine, morpholine, or R is phenyl or heteroaryl such as pyridyl, imidazolyl, pyrimidinyl, and the like.

The present invention further relates to a method for the preparation of BPi salt in a one-pot two-step reaction comprising reacting tris(trimethylsilyl)-phosphite with borane-dimethylsulfide complex of the formula $BH_3.SMe_2$, reacting the intermediate **11** (see Scheme 2) with the desired base in water or in methanol, thus obtaining the corresponding salt of Bpi in very high yield.

In one embodiment, the intermediate **11** is treated with methanolic ammonia or with an aqueous NH_4OH solution, thus resulting in the ammonium salt of Bpi, **2a.**

In another embodiment, the intermediate **11** is treated with tributylamine, Bu_3N, in methanol, thus resulting in the Bu_3NH^+ salt of Bpi, **2b.**

In a further embodiment, the intermediate **11** is treated with triethylammonium bicarbonate buffer, thus resulting in the Et_3NH^+ salt of Bpi, **2c.**

The present invention further relates to the use of the boranophosphate salts of the invention as fertilizers, in detergent formulations, as additive in melts for the glass industry, in boron neutron capture therapy (BNCT) of cancer, and as synthetic building blocks in the synthesis of boranonucleotides that may be used for all the uses known today and that may be discovered in the future for boranonucleotides of various lengths.

DESCRIPTION OF PREFERRED EMBODIMENTS

We present here the preparation, characterization, and unique chemical properties of inorganic boranophosphate (BPi) salts. In addition, we demonstrate that Bpi ion is an excellent mimic of inorganic phosphate.

BPi was characterized by X-ray crystal structure, IR, and ^{1}H and ^{31}P NMR. The acid-base character of BPi was evaluated by the determination of acidity constants. Likewise, we evaluated the stability of BPi at various pH values, and calculated the decomposition rate constants at highly acidic pH. We also monitored the clustering of BPi in non-aqueous solutions, and the interconversion of the H-bonded clusters. Finally, we explored the chemical behavior of BPi with respect to various organic and inorganic reagents. BPi is stable under the following conditions: both basic and acidic pH ($pH > 2$); in the presence of amines; and in the presence of Mg^{2+} ions. However, a P-B bond cleavage is observed upon the reaction of BPi with carbodiimides or upon catalytic hydrogenation. The reducing nature of the BH_3 moiety is drastically decreased in BPi. Likewise, the nucleophilicity of BPi's oxygen vanishes as compared to Pi.

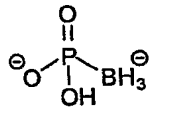
Based on its water solubility, acid-base character, and H-bonding properties, BPi appears as a perfect mimic of Pi and is an attractive alternative to the known phosphate isosters. Furthermore, based on BPi's unique chemical properties, numerous applications can be envisaged for this novel molecule.

As mentioned above, the field of boranophosphates deals extensively with the related nucleotide/oligonucleotide analogues. However, to the best of our knowledge, no attention has been given to the unique and chemically interesting inorganic

boranophosphate **2**, BPi, which may have numerous promising applications ranging from biochemical probing to modulation of materials properties.

The existence of BPi in the free form, BH_3O_3P (CAS No. 178449-22-4), has been detected previously by Li et al. in 1996 (Ref. 20), while carrying out the hydrolysis of thymidine boranomonophosphate in neutral solution. The compound was not stable: its NMR was determined in the solution, and it decomposed before it could be isolated.

Although the related dimethyl boranophosphate potassium salt, **3**, has been described by Imamoto *et al.*[13] and by Wada and Saigo,[14] the preparation of stable salts of inorganic boranophosphate **2**, has not been reported to our knowledge.



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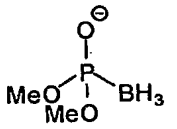

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The invention will now be illustrated by the following non-limitative examples.

EXAMPLES

Experimental

(i) General. All air- and moisture-sensitive reactions were performed in flame-dried, nitrogen flushed flasks sealed with rubber septa, and the reagents were introduced with a syringe. The progress of the reactions was monitored by TLC on precoated Merck silica gel plates (60K-254). Column chromatography was performed with Merck silica gel 60 (230-400 mesh). Compounds were characterized by nuclear magnetic resonance

using Bruker DPX-300, DMX-600, or AC-200 spectrometers. NMR spectra were recorded on a Bruker AC-200 spectrometer with a ^{31}P NMR probe (isotope frequency of 81 MHz) using 85 % H_3PO_4 as an external reference. IR spectra of BPi in KBr pellets were recorded on Nicolet Impact 400D spectrometer using OMNIC program. IR spectra of BPi in solution were measured using a BRUKER Vector 22 equipped with an liquid nitrogen cooled MCT detector. For the ATR measurements, a Harrick variable angle ATR accessory was used. For one spectrum, 100 scans were coadded at a resolution of 4 cm^{-1}. The clean ATR Germanium crystal (Harrick Scientific Corporation) was measured for the background spectra (cutoff 680 cm^{-1}). The machine is a Nonius KappaCCD diffractometer data were collected at 120K with scans of 1 deg collected at a speed of 1deg/20sec the merging R-factor on the data was 0.046 with 36867 reflections collected and 2979 unique. The crystals were colorless needles. Melting points were measured using Fisher-Johns melting point apparatus. Apparent pH values were measured with Hanna Instruments pH-meter (HI 8521) equipped with an Orion micro-combination pH electrode (9802).

(ii) Synthesis of dibenzyl boranophosphate 9. The synthesis was carried out according to Scheme 1B hereinafter. To a solution of dibenzylphosphite (300 μL, 1.186 mmol) in dry THF *N*, *O*-bis(trimethylsilyl)acetamide (880 μL, 3.56 mmol) was added with pippetor and the mixture was stirred for 25 min at room temperature. The solution was cooled to 0 °C, and 2M $BH_3 \cdot SMe_2$ complex in THF (2.9 mL, 5.8 mmol) was added. The solution was stirred at room temperature for 15 min, and then evaporated. 24 % NH_4OH solution (6 mL) was added and the mixture was stirred at room temperature for 1h, and then freeze-dried. The product was purified by silica gel column chromatography

(elution with $CHCl_3$: MeOH, 12:1) and obtained as colorless oil in 71 % yield (231 mg, 0.84 mmol). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.22 (s), 4.86 (m), 0.3 (1-1-1-1 quartet) ppm. ^{31}P NMR ($CDCl_3$, 81 MHz): δ 97.5 (q) ppm. MS FAB (negative) m/z: 275.140 (M^-).

(iii) Synthesis of inorganic boranophosphate 2a. The synthesis was carried out according to Scheme 2 hereinafter. To a solution of tris(trimethylsilyl)phosphite (600 μL, 1.795 mmol) in dry CH_3CN (5 mL) under N_2 at 0 °C, 2 M $BH_3{\cdot}SMe_2$ complex in THF (1.35 mL, 2.7 mmol) was added. The resulting solution was kept at room temperature for 15 min. Dry MeOH (15 mL) and 2 M NH_3 in EtOH (1.8 mL, 3.6 mmol) were added and the mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure and the product was obtained as a white solid in 93 % yield (202 mg, 1.556 mmol), mp > 240 °C. ^1H NMR (D_2O, 200 Hz): δ 0.27 (d of 1-1-1-1 quartet, J_{PH} = 22, J_{BH} = 87 Hz, 3 H). ^{31}P NMR (D_2O, 81 MHz): δ 80.38 (1-1-1-1 quartet, J = 156 Hz, 1-1-1-1-1-1-1 septet, J = 52 Hz) ppm. IR (KBr): ν 2412, 2378, 2352, 1181, 1149, 1077-903, 654 cm^{-1}.

Compound **2a** was converted to the corresponding tetraethylammonium salt as follows: **2a** was passed through a Sephadex-CM C-25 – tetraethylammonium-form column (prepared from the corresponding sodium form resin upon loading with excess Et_4NCl) and the column was washed with ca. 20 volumes deionized water. The solution was freeze dried to yield tetraethylammonium BPi, **2d**, as a white solid. Based on the pH of **2d** solution and the ^1H NMR the expected molecular formula is $BH_3O_3PH_{1.5}(Et_4N)_{1.5}$. Elemental analysis: %H calcd. 11.9, found 11.3; %P calcd. 10.7, found 9.5.

(iv) Synthesis of inorganic boranophosphate 2b. The tributyl ammonium salt of the inorganic boranophosphate was prepared as described above for **2a**. However, Bu_3N

(0.85 mL, 3.57 mmol) was added instead of NH_3/EtOH. The product was obtained as a white solid in 93 % (645 mg, 1.385 mmol), mp 83-84 °C. IR (KBr) ν: 2407, 2381, 2350, 1184, 1150, 1100-850, 655 cm^{-1}.

(v) Determination of pK_a value of inorganic boranophosphate 2a. The pK_a values of **2a** were evaluated by ^{31}P NMR at room temperature. Solutions of **2a** (0.15-0.18 M) at different pH values were prepared by the addition of dilute sodium hydroxide or hydrochloric acid solutions. ^{31}P NMR chemical shift was monitored as a function of the pH. A five-parameter sigmoid function was fitted to the data using Sigma Plot 2000 (SPSS, Inc.):

$$\delta = \delta_0 + a / (1 + e^{-((pH-pH_0)/b)})^c$$

The inflection point, which is determined by the second derivative of the fitted sigmoid function, is the pK_a value.

(vi) Determination of the decomposition rate of BPi 2a at pH 2. The stability of **2a** in acidic solution was evaluated by ^{31}P NMR at room temperature, monitoring the formation of the deboranation product – H-phosphonate. A 0.16 M solution of **2a** at pH 2 was prepared by the addition of dilute hydrochloric acid to a solution of inorganic boranophosphate (NH_4^+ salt) in H_2O and 10 % D_2O. The percentage of decomposition of **2a** is based on integrations of the PBi signal (90.93 ppm) and the H-phosphonate signal (3.3 ppm). The decomposition rate was determined by measuring changes in the integration of the respective NMR signals with time, within 96 h.

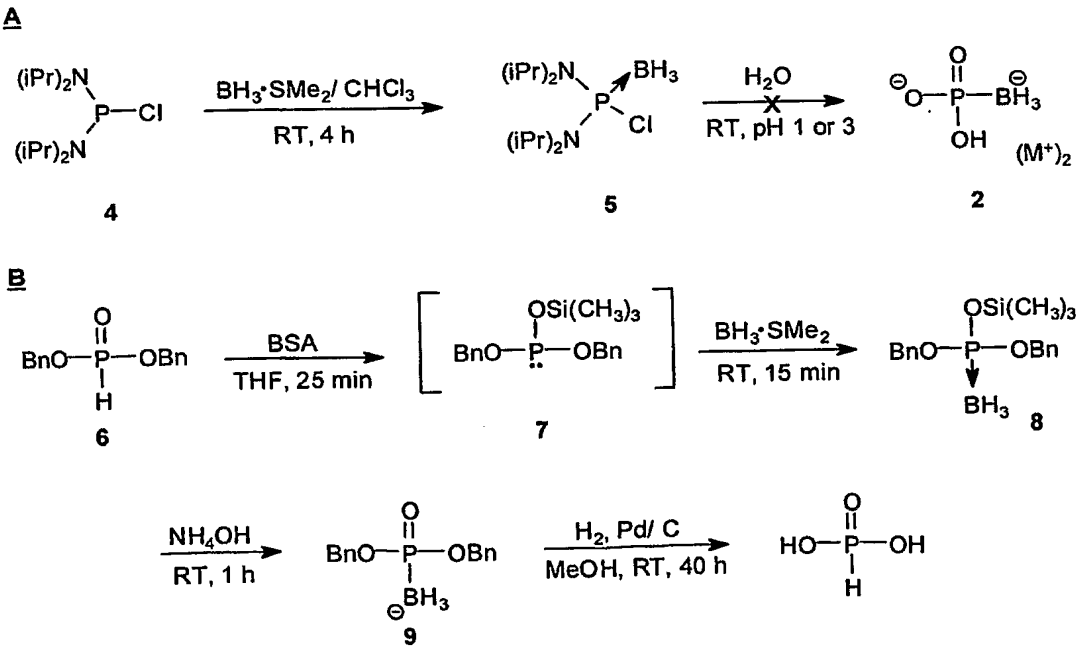
EXAMPLE 1. Synthesis of BPi salts

For the preparation of BPi salts, we first attempted the treatment of bis(di-isopropylamino) chlorophosphine **4** with borane dimethylsulfide complex,[15] followed by

acidic hydrolysis (pH 3 or 1) for several hours, according to **Scheme 1A**. This attempt resulted in a mixture of several phosphorus species but BPi was not obtained..

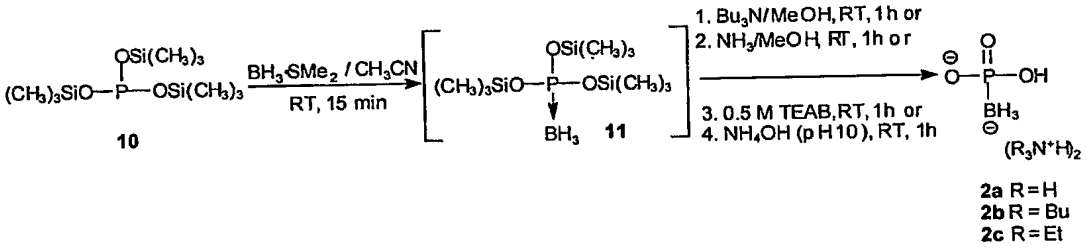
In an alternative approach, depicted in **Scheme 1B**, dibenzylphosphite **6** was treated with bis(trimethylsilyl)acetamide in THF, followed by boronation of the silylated intermediate **7** with BH_3.dimethylsulfide complex. Finally, hydrolysis of compound **8** with concentrated ammonium hydroxide for 1 h, resulted in dibenzyl boranophosphate **9** in 71% overall yield (see Experimental, (ii)). However, all attempts to remove the benzyl groups by either catalytic hydrogenation or acidic hydrolysis, pH 1.3, resulted in the cleavage of the P-B bond, leading to phosphorus acid instead of BPi.

Scheme 1



In a further attempt, we were able to obtain BPi in an excellent overall yield in a two step one-pot reaction starting from tris(trimethylsilyl) phosphite[16] **10** , as depicted in Scheme 2 (see Experimental (iii)). Phosphite **10** was treated with BH_3·dimethylsulfide complex in dry acetonitrile under inert atmosphere for 15 minutes. Subsequently, intermediate **11** was treated with 2 M methanolic ammonia for 1 h to give the ammonium salt BPi **2a**, as a white solid in 93% yield based on ^{31}P NMR spectroscopy. No further purification was conducted, since volatile silyl derivatives and excess of BH_3:SMe_2 complex were removed by evaporation. Alternatively, intermediate **11** was treated with $NH_4OH_{(aq)}$ solution (pH 10), or tributylamine (Bu_3N) in MeOH, or 0.5 M triethylammonium bicarbonate buffer (pH 7.5) and freeze dried or evaporated to provide the corresponding BPi salts **2a**, **2b** (see Experimental, (iv)), or **2c**, respectively.

Scheme 2



Product **2a** was highly water-soluble, whereas **2b** dissolved only in organic solvents such as – MeOH, CH_3CN, DMF, and $CHCl_3$. Product **2c** was highly soluble both in water and in organic solvents.

EXAMPLE 2. Characterization of Compounds 2a and 2b

(i) NMR of Compound 2a

Compound **2a** in water was characterized by ^{31}P NMR showing a signal at ca. 80 ppm (Fig. 1). Inorganic boranophosphate ^{31}P NMR spectrum shows a typical pattern

including two overlapping signals: one of them is a large 1-1-1-1 quartet, due to coupling of P to ^{11}B isotope (spin $I = 3/2$), and a smaller pattern composed of a 1-1-1-1-1-1-1 septet, due to coupling with ^{10}B isotope ($I = 3$). The relative height of the latter peak is 0.14 of the former one (Fig. 1A). This ratio is due to the 80:20 relative natural abundance of the isotopes, and the gyromagnetic ratio ($\gamma(^{11}B) / \gamma(^{10}B) = 2.99$).[17] BPi's hydrogen-coupled ^{31}P NMR spectrum, showed further splitting of the lines into a quartet (Fig. 1B). ^{1}H NMR spectrum showed a typical *dq* pattern, resonating at ca. 0.2 ppm, due to coupling of H to both ^{11}B isotope and P atom (Fig. 1C). This pattern overlaps a more complex pattern due to coupling of H to both ^{10}B and P.

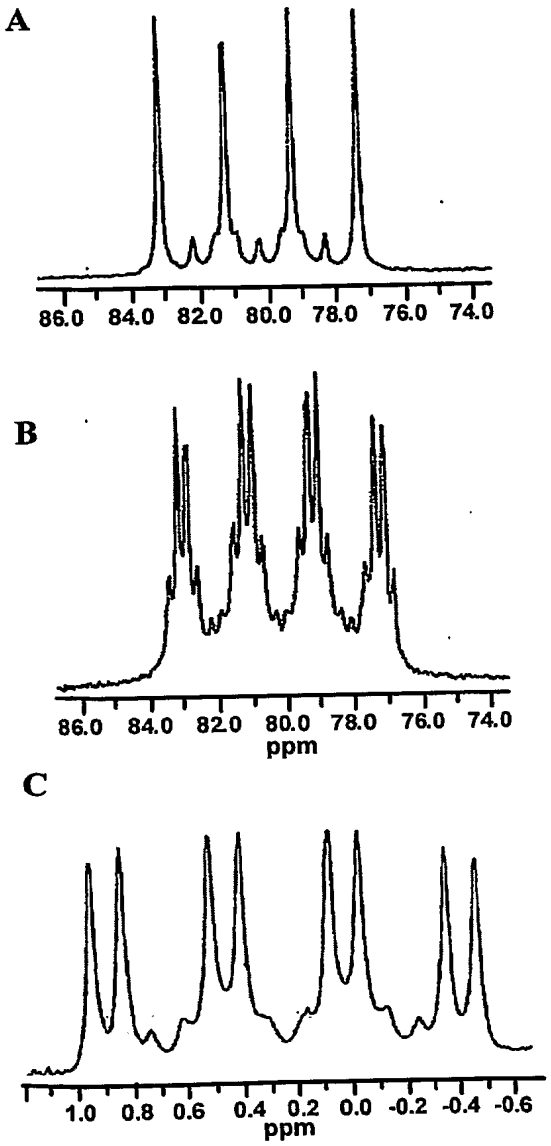


Figure 1. NMR spectra of BPi. A. ^{1}H decoupled – ^{31}P NMR spectrum in D_2O, 200 MHz. B. ^{1}H coupled – ^{31}P NMR spectrum in D_2O, 81 MHz. C. ^{1}H NMR spectrum in D_2O, 200 MHz

The chemical shift of BPi is pH-dependent. For instance, at pH 4.87 and 13.20 the phosphor resonates at 84 and 63 ppm, respectively (Fig. 2). Likewise, the P-B coupling constant is also pH-dependent, and is reduced as pH decreases (e.g. 147 and 183 Hz for pH 4.87 and 13.2, respectively). This pH-dependent J value indicates structural changes of BPi, which are due to the reduction of O-P-O angles upon protonation of the molecule.

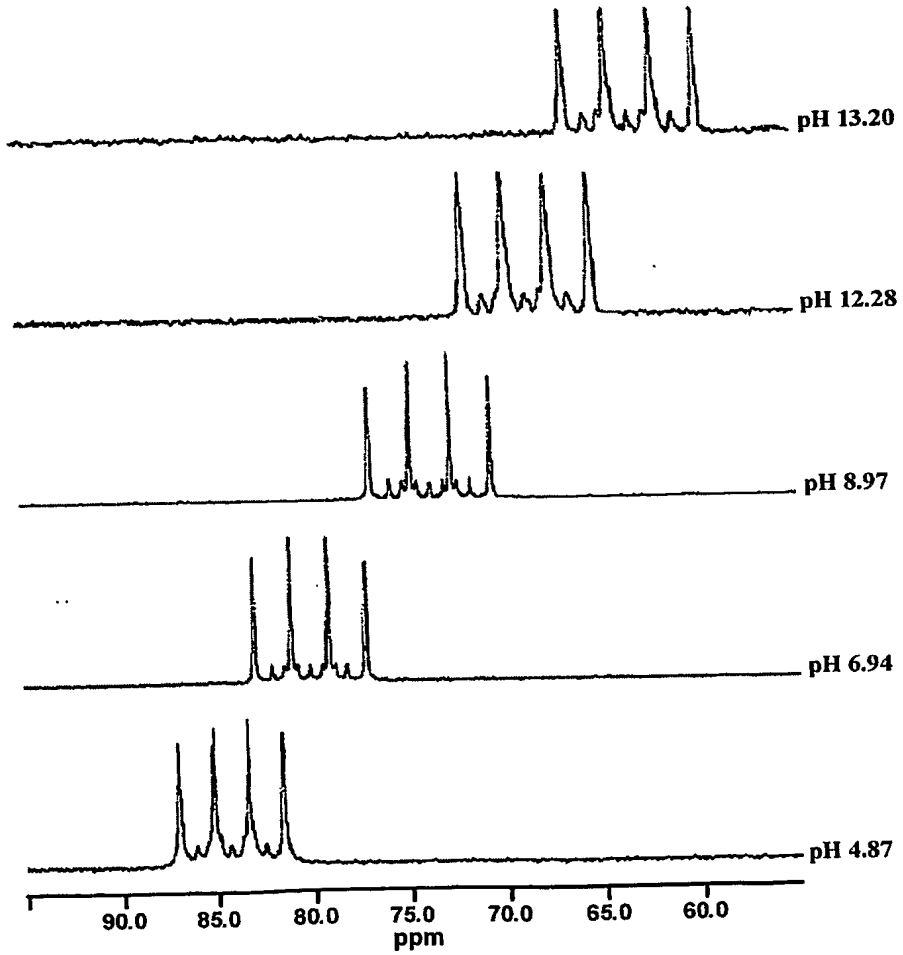


Figure 2. pH-dependent ^{31}P NMR chemical shift of BPi in H_2O within the pH range 4.87-13.20, 81 MHz

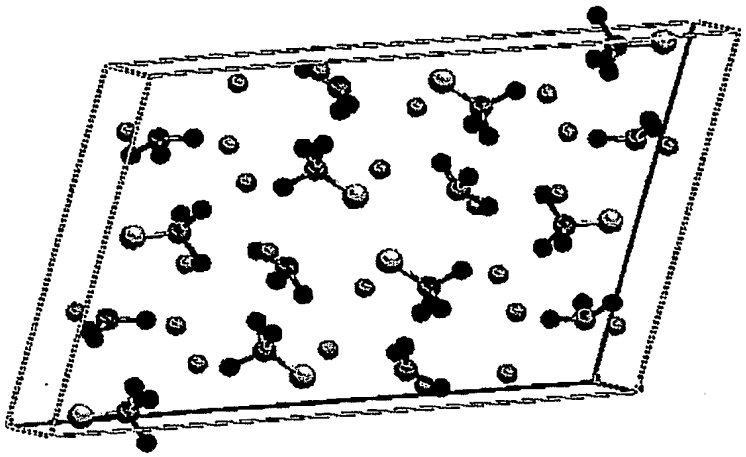
(ii) X-ray crystallography of 2a

To obtain structural information on BPi, compound **2a** was crystallized from aqueous solution (pH 7). In addition to compound **2a**, the crystal contained phosphorus acid in a 1:1 ratio. This unexpected ratio did not reflect the molar ratio in the original BPi solution in which phosphorus acid was less than 5%.

The cell unit contains 8 BPi ions, 8 H-phosphonate ions, and 24 ammonium ions (Fig. 3A). Apparently, for each BPi anion, one ammonium counter-ion is observed at a distance of 2.74 Å from oxygen O1A, whereas two ammonium counter-ions are observed around H-phosphonate.

For BPi, an average typical length of P-B bond was measured, 1.892 Å, whereas for the three P-O bonds, the average lengths were: 1.585 and 1.605 Å, and 1.524 Å, respectively (Fig. 3B). A deviation from tetrahedral angles was observed with values of 111-118 ° for B-P-O and 104-105 ° for O-P-O angles.

A



B

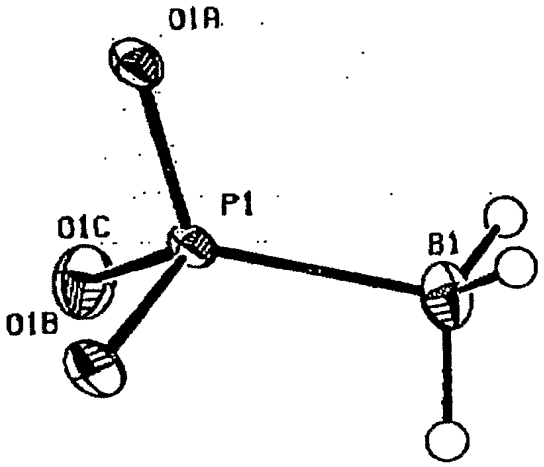


Figure 3. X-ray crystal data for BPi. A. cell unit includes 8 BPi molecules, 8 H-phosphonate molecules, and 24 ammonium ions. Crystal data of **2a**: monoclinic, *P2(1)/c*; a = 23.616(5) Å, b = 6.3470(13) Å, c = 15.325(3) Å; V = 2172.9(8) Å^3; Z = 12; D_{calc} = 1.623 g/cm^3; F(000) = 1104; 3094 reflections collected, R = 0.1015, Rw = 0.2345, GOF = 1.286. B. ORTEP drawing of BPi. Selected bond distances (Å) and angles (deg): P(1)-O(1A), 1.524(7); P(1)-O(1B), 1.617(7); P(1)-O(1C), 1.583(7); P(1)-B(1), 1.891(11);

O(1A)-P(1)-O(1C), 104.0(4); O(1A)-P(1)-O(1B), 105.3(4); O(1C)-P(1)-O(1B), 104.4(4); O(1A)-P(1)-B(1), 118.2(5); O(1C)-P(1)-B(1), 113.0(5); O(1B)-P(1)-B(1), 110.7(5)

Comparison with X-ray crystal data obtained for the related dimethyl boranophosphate salt,[13] 3, indicated similar values for B-P and O-P bond lengths: 1.895, 1.490, 1.597 and 1.612 Å, respectively. For dimethyl boranophosphate, one potassium ion was found near one of the oxygen atoms at a distance of 2.66 Å. Based on a comparison of the bond lengths of dimethyl boranophosphate salt with BPi, we assume that the BPi bears two H atoms, which are not shown in the crystallographic data.

The shortest P-O bond, 1.524 Å, indicates a partial double bond character, and is in accordance with values found in the structures of phosphate diesters (1.47-1.51 Å) and monoesters (1.49-1.53 Å). This P-O bond is significantly longer than the bond observed in phosphate triesters (1.38-1.44 Å).[18]

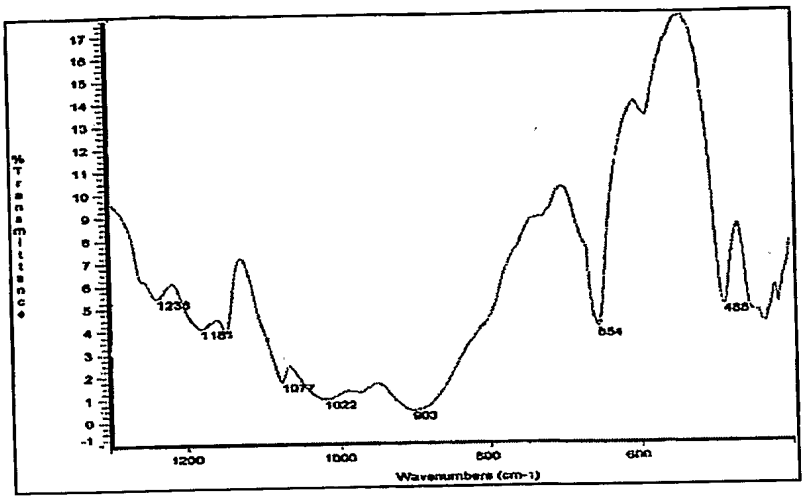
(iii) Infra-red (IR) spectrum of 2a and 2b

IR spectrum of either **2a** or **2b** in KBr pellet indicated characteristic bands for P-B and B-H in addition to bands associated with P-OH and P=O (Fig. 4A). Specifically, three absorptions at 2350, 2381, 2407 cm^{-1} (*s*) (not shown) correspond to B-H stretches, and one absorption for P-B stretch was observed at 654 cm^{-1} (*m*).[19] Typical absorptions were observed for P-OH and P=O stretches, 900-1080 cm^{-1}, and at 1140-1250 cm^{-1}, respectively.

For an evaluation of solvents' effects on H-bonds between BPi ions, IR spectra of BPi, **2a**, in aqueous and methanolic solutions (see below 'H-bonding of BPi') were measured in a germanium cell and compared to the corresponding spectrum of the neat compound (Fig. 4B). Comparison of those spectra indicated only minor differences. For

instance, a ca. 10 cm^{-1} shift to lower frequencies was observed for P=O stretch of BPi, neat or in MeOH, as compared to BPi in aqueous solution. This shift is probably due to H-bonding based clustering under the former conditions. The typical fine-structure for P=O stretch in BPi neat in the range of 1144-1178 cm^{-1}, which is possibly also due to H-bonded clusters, is lost in water. The corresponding spectrum in MeOH appears like an average of the neat and aqueous solution spectra, probably indicating the presence of both BPi clusters and solvent H-bonded species.

A



B

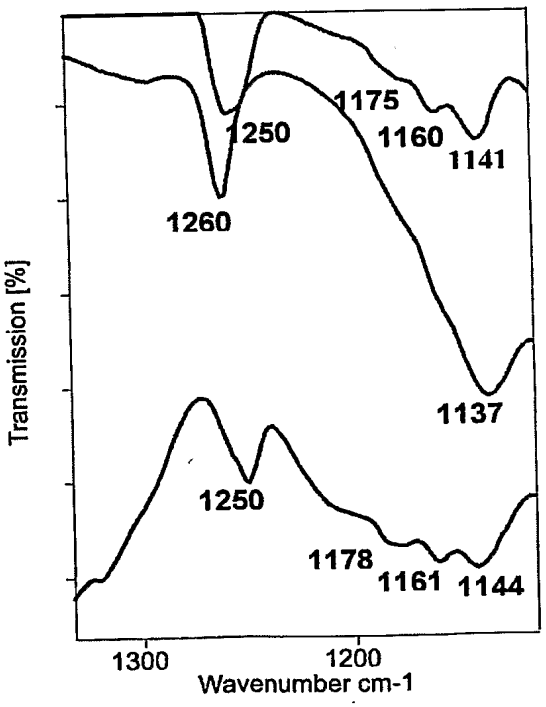


Figure 4. IR spectra of BPi. A. IR spectrum of **2a** (KBr pellet; 1300-400 cm^{-1}). B. IR spectra of **2c** (germanium cell; 1300-1100 cm^{-1}; cutoff: 680 cm^{-1}): green line- methanolic solution, red line - aqueous solution, black line – neat.

(iv) Chemical properties of 2a, 2b and 2c

(a) Acid-base properties. The acid-base character of BPi was studied by ^{31}P NMR – monitored pH-titration , as described in Experimental. (v). The chemical shift of compound **2a** was plotted against pH (Fig. 5A). For the pH range of 4.8 - 13.2, two inflection points were observed. The second derivatives of the fitted function provided two pK_a values: 7.12 (Fig. 5B) and 12.54, with R^2 values of 0.999 and 0.997, respectively. These values are similar to the corresponding values of the second and third protonation equilibria of phosphoric acid (7.21 and 12.67) and are higher than those for phosphorous acid (H-phosphonate; 1.8 and 6.2). The results are shown in Fig. 2.

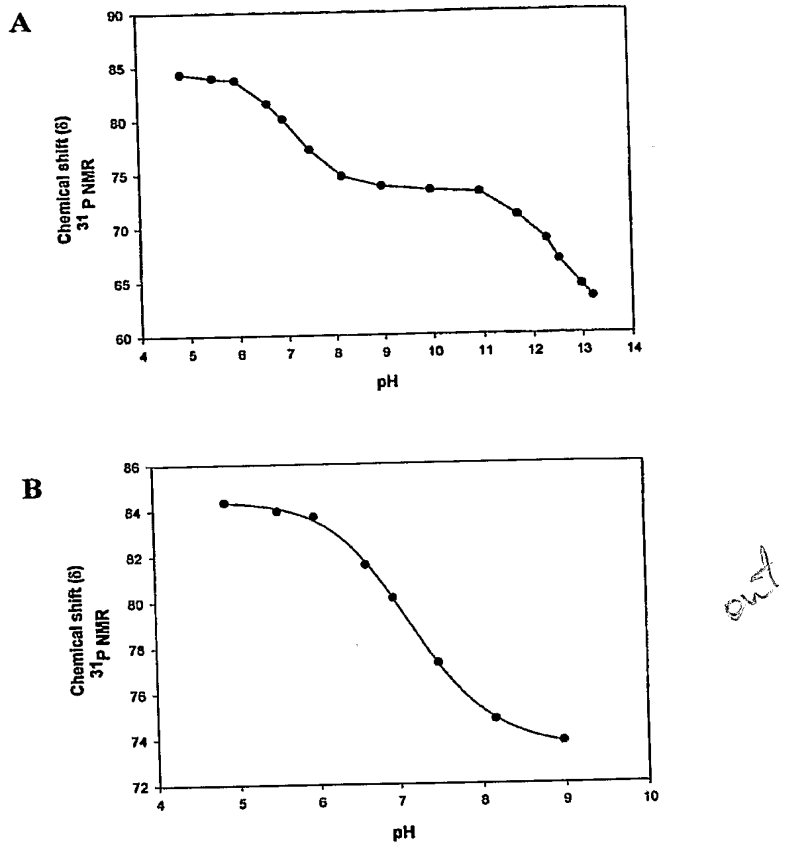
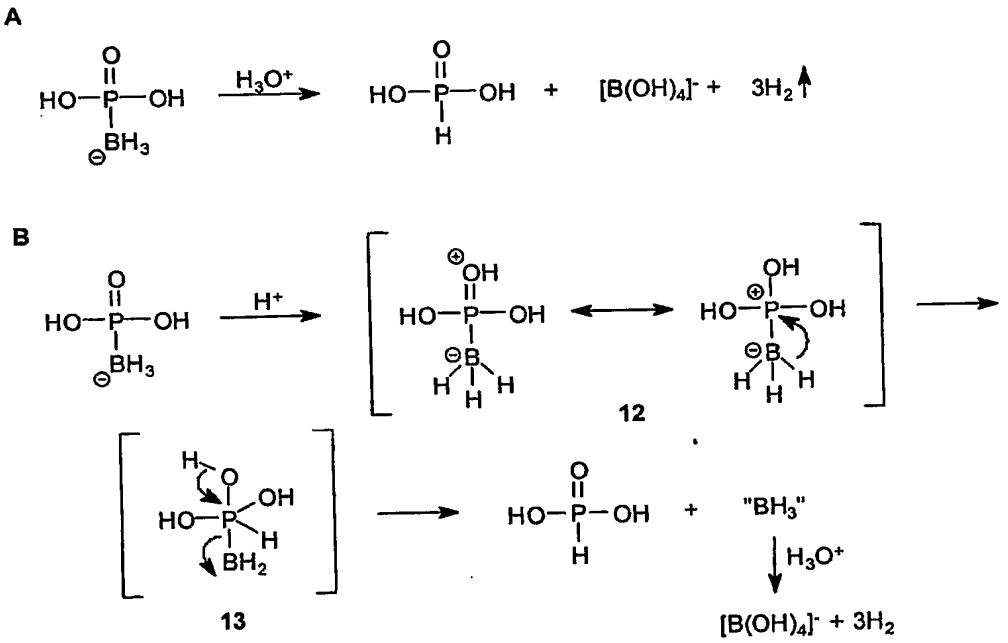


Figure 5. Determination of pK_a values of BPi. Plot of BPi's ^{31}P NMR chemical shift in H_2O as a function of pH. A. Two inflection points are observed in the pH range of 4.87-13.20. B. First pK_a value, based on the second derivative of the fitted graph, is 7.12.

(b) Stability of BPi. The stability of BPi was determined as described in Experimental, (vi). As shown above, BPi is stable in neutral and basic solutions. For instance, after 48 h at room temperature at pH 13.7, no degradation of BPi was observed by ^{31}P NMR. BPi is also relatively stable in acidic solution at pH > 2. At pH 2, BPi was degraded slowly to phosphorus acid at a rate of $7x10^{-7}$ sec^{-1}, R^2 = 1.00 ($t_{1/2}$ 275 h), as monitored by ^{31}P NMR.

Under highly acidic conditions, evolution of H_2 is clearly observed, the P-B bond is cleaved, and boric acid is formed together with phosphorus acid (Scheme 3A).[20] The mechanism possibly involves the intermediacy of a phosphonium ion, **12**, that is subsequently neutralized by intra-molecular hydride migration. The putative resulting phosphorane intermediate, **13**, is unstable and eliminates BH_3, thus forming H-phosphonate (Scheme 3B). Phosphorus acid (H-phosphonate) was observed in ^{31}P NMR as a doublet at 3.5 ppm, J = 633 Hz. The borane reacts with water to liberate hydrogen gas and boric acid.

Scheme 3



As mentioned above, the inorganic boranophosphate (free BPi), resulting from neutral hydrolysis of thymidine 5'-boranomonophosphate at 60°C, was not stable.[20]

(c) H-bonding of BPi. Solutions of **2b** or **2c** in organic solvents (MeOH, DMF, CH_3CN, or $CHCl_3$), showed unexpected ^{31}P NMR spectra. Product **2b** in MeOH apparently consisted of three different but pattern-related signals. The signals with chemical shifts of 80.0 (A), 86.2 (B), and 90.8 (C) ppm, each had an identical BPi-typical–pattern (Fig. 6. **B**

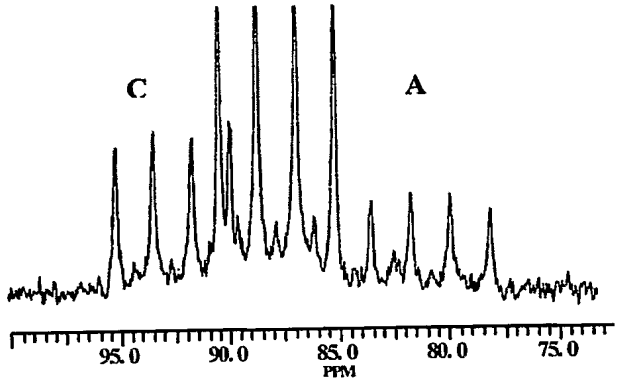


Figure 6. ^{31}P NMR spectrum of BPi **2b** in methanolic solution

Several minutes after the dissolution of **2b** in MeOH, signals A, B, and C were observed in the ^{31}P NMR spectrum, with A and B as the major peaks (C constitutes ca. 5% of the mixture). The composition of the initial mixture is time-dependent due to interconversion of the species. Monitoring of this process in CD_3OD for 160 h at room temperature, indicated the conversion of A and B to C, with a final ratio of C:B 4.4:1 (A completely disappeared). ^1H-coupled ^{31}P NMR spectrum indicated quartet splitted signals for B and C, namely, no D-H exchange occurred.

A spectrum similar to that shown in Fig. 6, and time-dependent interconversion of the species, were observed for **2b** also in DMF, CH_3CN, and $CHCl_3$.

The possibility that the additional BPi-like species are the corresponding mono- or di-methyl esters, due to a reaction of **2b** with MeOH, was ruled out because their ^{13}C and ^{1}H NMR spectra in $CDCl_3$ were devoid of a methyl ester signal.

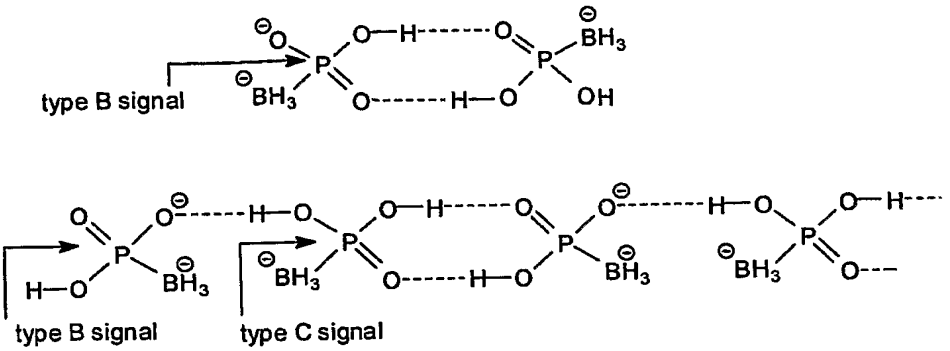
The three signals seen in ^{31}P NMR of **2c** in organic solvents, converged into one, probably A, δ 79.8 ppm, after solvent evaporation and dissolution in D_2O. Therefore, the possibility that signals B and C are due to di-borano-pyrophosphate and tri-borano-trimetaphosphate anhydrides, resulting from **2c**, is less likely.

A significant amount of evidence has been accumulated over the past decades regarding strongly H-bonded networks in phosphoric acid and its derivatives.[21,22] Various clusters were reported ranging from zigzag chain to cyclic dimers and trimers.[21] Dimerization occurs in the solid state,[23] in highly concentrated phosphoric acid solutions,[22,24] in freons,[21b] or in organic solvents (e.g. DMF or acetonitrile).[25] Polymerization was reported for monoesters of phosphoric acid in benzene.[26]

To assess the possibility of observing separately different H-bond-clustered species on the NMR time-scale, we measured the ^{31}P NMR spectrum of the parent phosphate bis(tributylammonium) salt in benzene, where clustering is known to occur.[26] Indeed, three signals were clearly observed at 3.63, 3.23, and 2.93 ppm, demonstrating that H-bonded phosphate clusters can be detected by ^{31}P NMR. These three phosphate signals in benzene converged into one in acetonitrile and MeOH.

Based on the tendency of phosphoric acid (and its derivatives) to form dimers and polymers in organic solvents,[26,27] we propose that signal A corresponds to the BPi monomer and signals B and C correspond to H-bond based clusters of BPi (Scheme 4 and Discussion). These clusters are not large, < 30Å, based on light scattering measurements.

Scheme 4



(d) Reactions of BPi with selected reagents. The reactivity of BPi towards various organic and inorganic reagents was explored as part of the characterization of BPi's chemical nature. These reagents include: tosyl chloride, phosphorous oxychloride, a carbodiimide, pyridine and imidazole, H_2, Zn^{2+} and Mg^{2+} ions.

A carbodiimide reagent is used for the condensations of phosphate and its derivatives with another phosphate derivative to provide the corresponding phosphoric anhydride. The reaction of **2a** with an excess of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) was explored in water (pH 6.5), 4 h at 37°C. The addition of 3 eq of EDC to BPi resulted in excessive loss of this compound, due to complete P-B bond cleavage of **2a**, to yield phosphorus acid (72 % of **2a** was degraded after 4 h, based on ^{31}P NMR). This finding is in contrast to diethylphosphite (cyano- or carbomethoxy)borane analogues, that are stable to carbodiimide (DCC).[28]

The P-B bond was also found to be sensitive to catalytic hydrogenation. Thus, when compound **9** was subjected to hydrogenation (over Pd/C), the P-B bond, was also reduced, yielding phosphorus acid.

The reactivity of BPi with imidazole and pyridine was studied. Specifically, a solution of BPi with 2 or 10 eq of imidazole in CD_3OD remained unchanged for 96 h, based on ^{31}P NMR spectra. Likewise, only a negligible cleavage of the P-B bond was observed after 113 h for a solution of BPi in pyridine.

BPi is apparently more stable to imidazole and pyridine than the related analogue, tetramethyl boranopyrophosphate. The reaction of 5'-DMT-2'-deoxy-thymidine with tetramethyl boranopyrophosphate in the presence of N-Me-imidazole was reported to proceed with the partial removal of the borane group. Likewise, when pyridine was used as a solvent, partial removal of the borane group was observed. However, Et_3N or iPr_2EtN did not cause any loss of the borane group.[29]

The presence of divalent metal ions such as Zn^{2+} and Mg^{2+} in DMF and water for 48h and 4h, respectively, left BPi unchanged.

Dimethyl boranophosphate monopotassium salt, **3**, is a nucleophile reacting in an S_N2 manner with alkyl halides, acid chlorides, etc.[13] Thus, the reaction of **3** with mesyl chloride resulted eventually in the complete conversion to tetramethyl boranopyrophosphate.[13] However, we found that BPi salt **2** demonstrates an entirely different chemical reactivity. Whereas analogue **3** plays the role of an efficient nucleophile, the related BPi is entirely a non-nucleophile. Thus, when BPi was treated with tosyl chloride or mesyl chloride (with or without amine) in acetonitrile for 24 h, even at 60° C, no reaction occurred. Likewise, the reaction of BPi with phosphorus oxychloride and its derivatives ($P(O)Cl_2R$) yielded no product.

Discussion

The quest for phosphate bioisosters over the last several decades included phosphonates, and later, α-halo phosphonates.[30] These α-halo phosphonates, e.g. difluoromethyl-phosphonates, were proposed as isosteric and isopolar analogues of the parent phosphates.[30,31] Another widely used isoster of phosphate is phosphorothioate and its analogues.[4]

Spielvogel and Ramsay-Shaw proposed boranophosphate analogues as bioisosters of natural nucleotides based on their unique properties.[6] Specifically, the borane moiety is isoelectronic, and isosteric with oxygen. Yet, this moiety is incapable of coordinating metal ions or forming H-bonds.[32] Thus, boranophosphate analogues have a different charge distribution and polarity than the corresponding natural nucleotides.[33] Despite the extensive study of boranophosphate nucleoside analogues, the exploration of the parent inorganic boranophosphate has not been reported.

The various potential applications of a phosphate isoster, together with the limitations of the currently available isosters, justify the continued search for the perfect inorganic phosphate mimic.

Therefore, the unique and chemically interesting inorganic boranophosphate, **2**, has been investigated here as a mimic of phosphate with respect to properties such as: water solubility, geometry, acid-base character, and H-bonding.

BPi ammonium salt, **2a**, which is prepared in excellent yield in a one-pot, two-step reaction from tris(trimethylsilyl)-phosphite, **10**, is highly soluble in water, like the parent phosphate, Pi.

The geometry of BPi (**2a**) has been determined by X-ray crystallography. Except for the long P-B bond (1.892Å) and B-P-O angles that are slightly larger than tetrahedral angles, P-O bond lengths and angles of BPi are in accordance with those in the parent compound (Fig. 3B).

The great similarity of BPi to inorganic phosphate, Pi, is demonstrated here also by comparing their pK_a values. Essentially, the acid-base character of BPi is not altered in comparison to Pi. This finding is in contrast to the corresponding phosphorothioate isoster where there is a reduction of ca. 2 log units in the acidity, in comparison to phosphate.[34] Likewise, pK_{a2} values of α-mono- and di-fluorophosphonate isosters are 1 and 2 log units, respectively, lower than pK_{a2} of phosphoric acid.[31]

Indications for H-bond based clustering of BPi in various organic solvents were obtained from IR and ^{31}P NMR spectra (Figs. 4 and 6).

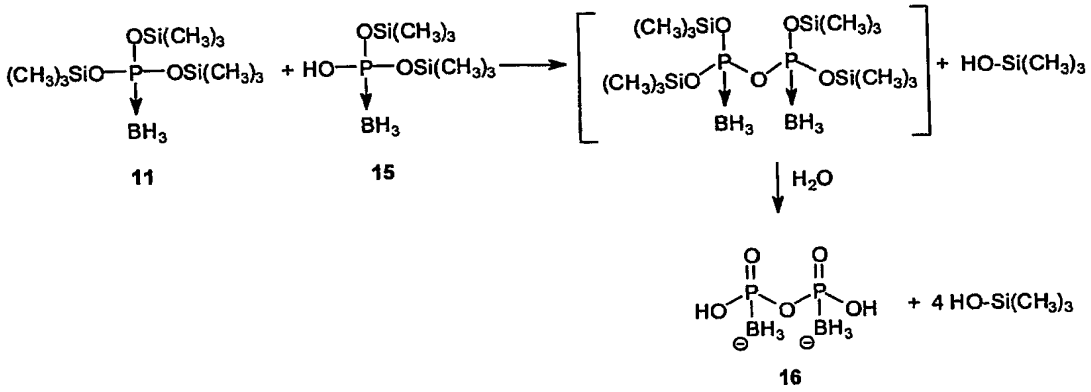
The three highly related BPi-typical signals observed for BPi in ^{31}P NMR spectra in organic solvents (e.g. in MeOH, Fig. 6), could be considered to result either from related covalently bound species, namely, anhydrides, or, non-covalently bound species, namely, H-bonded clusters (Scheme 4).

The formation of BPi anhydrides is considered unlikely, since the conditions required for their formation are not fulfilled here. Specifically, formation of the parent phosphoric acid anhydrides requires drastic conditions (up to 250°C at very low pH, or the presence of a condensation agent).[35] Obviously, these conditions do not exist in BPi's NMR samples.

Alternatively, one can consider anhydrides' formation during the preparation of BPi. Hypothetically, a partially methanolized/hydrolyzed intermediate **15**, could react

with the silylated intermediate **11** to provide the corresponding BPi anhydride, **16** (Scheme 5).

Scheme 5



Yet, the hydrolysis of condensed phosphates is known to be quite slow (i.e. several weeks at 60°C and pH 5).[36] Since ^{31}P NMR spectra of **2c**, exhibiting several signals in organic solvents, showed only one signals when measured in water, the existence of BPi anhydride is ruled out. Furthermore, **2a** or **2c**, were prepared in water, a medium in which no anhydride is formed. As such, **2a** and **2c** exist also as several species in organic solvents, based on their ^{31}P NMR spectra.

All these arguments are highly supportive of the clustering of BPi in organic solvents, as observed in ^{31}P NMR and IR spectra. The clusters mixture, formed instantaneously, slowly converges to the thermodynamically stable cluster, having a signal ratio of C:B 4.4:1 after 160 h.

Based on our observations on the pH-dependent chemical shift of BPi (Fig. 2), and on the determination of BPi's acidity constants (Fig. 5), we propose the following

assignment of signals A, B, and C. Signal A corresponds to the monomeric BPi, whose chemical shift at ca. 80 ppm indicates that half the BPi monomer population bears two protons, and the other half bears one proton (Fig. 5). Signal B at 86 ppm corresponds to a BPi moiety that has one BPi H-bonded neighbor. Namely, signal B could result both from BPi dimers and higher clusters (Scheme 4). In these cases, each BPi is associated with an additional proton. Therefore, the chemical shift of the BPi dimer shifts down field (86 ppm, as at pH 4.7, Fig. 2). BPi also forms clusters, as indicated by signal C corresponding to BPi moiety that has two H-bonded BPi neighbors (Scheme 4). A BPi moiety in the middle of a cluster is associated with three protons, resulting in an additional down field shift to 91 ppm, corresponding to that of BPi at pH 2 (data not shown).

Low H-bonded clusters (i.e. dimers and trimers) are formed almost instantaneously. This is probably the stage of nucleation. Once a critical nucleus is formed, a slow process of high-order clustering occurs. In this step the concentration of A in solution is drastically reduced. This H-bonded based clustering mechanism is also supported by the observation that upon the evaporation of the organic solvent from the species mixture and dissolution in water, only A is detected.

We speculate that a two-fold deriving force affects BPi's clustering. First, the lipophilic BH_3 moieties possibly form the core of the cluster due to hydrophobic interactions (in protic and polar organic solvents). Second, this core is further stabilized by P-$O^{-}\cdots$HO-P H-bonds.

This hypothesized mechanism is based on the following observations: a. H-bonded clusters can be clearly observed on the NMR time-scale (as we showed for clusters of Pi in benzene) b. Pi forms clusters only in benzene. However, BPi forms

clusters even in CH_3CN and MeOH, implying that BH_3 plays a role in the pre-organization of the clusters.

The chemical behavior of BPi was also explored at various pH values, and with various organic and inorganic reagents.

BPi is stable under both highly basic and acidic conditions (at pH >2). In addition, BPi is stable in the presence of imidazole or pyridine, and in the presence of Mg^{2+} ions.

However, P-B bond cleavage is observed upon the reaction of BPi with carbodiimides. A loss of BPi's borane moiety also occurs at pH < 2. A mechanism involving hydride migration and phosphorane formation (Schemes 3 and 5) is perhaps responsible for the formation of H-phosphonate product in both cases.

Although BH_3, in complexes with a variety of sulfur/amine/oxygen compounds, is an efficient reducing agent, its reducing nature is drastically altered in BPi. For instance, while hydride transfer from "BH_3" to water occurs readily, BH_3 moiety in BPi transfers hydride only in a highly acidic solution (pH < 2). Likewise, while BH_3:THF complex readily reduces nitriles and amides to the corresponding amines,[37] the borane moiety in BPi is completely stable in acetonitrile and dimethyl formamide solutions.

The drastic alteration in the chemical nature of BPi is demonstrated also regarding the nature of Pi. While Pi is a nucleophile,[38] BPi is essentially a non-nucleophile. BPi does not react with electrophiles such as tosyl chloride and phosphorus oxychloride. The reason for this may be the electron withdrawing nature of the borane group which reduces the BPi oxygen's nucleophilicity.

The related dimethyl boranophosphate, **3**, was reported to be a good nucleophile.[13] The reason for the reduced BPi's nucleophilicity, as compared to **3**, might

be due to the "carboxylate-like" nature of the BPi as compared to the "alkoxide-like" nature of dimethyl boranophosphate.

Based on the geometry, water solubility, acid-base character, and H-bonding properties, BPi appears as a perfect mimic of Pi, and as an attractive alternative to the phosphorothioate and α-halophosphonate isosters.

Numerous applications can be envisaged for BPi as described hereinbelow and all of them are encompassed by the present invention.

Fertilizers - Among the essential elements required for plants growth are: P (macronutrient, 0.2 wt %) and B (micronutrient, 20 ppm). Likewise, K and N are also essential nutrients (1.0 and 1.5 wt %, respectively). Therefore, ammonium(or K^+ salt of BPi may be used for formulations of fertilizers. These salts provide the essential nutrients P, B, and N or K; are non-acidic, water-soluble, and have a high phosphorous content.

Detergents - BPi can be used for specialized detergent formulations. Here, BPi should be provided in the form of the corresponding pyro-phosphate or tri-phosphate. Such oligo-boranophopsphates are expected to soften hard water by sequestering unwanted Ca^{2+}/Mg^{2+} ions. The high charge on the phosphate chain helps to stabilize detergent micelles (as a 'builder'). Oligo-boranophosphates provide the correct pH for cleaning (slightly basic). Furthermore, in warm/hot water ($\geq$ 50° C), boric acid is produced from hydrolysis of BPi and exerts its effect as a bleaching agent.

Glasses - BPi may be an interesting additive in melts for glass making. For comparison, high quality borophosphate chemically durable optical glasses is obtained from: $MgO/Al_2O_3/K_2O/B_2O_3/P_2O_5$ melts.

Boron Neutron Capture Therapy (BNCT) - Boron-10 absorbs thermal neutrons. On capturing a neutron, ^{10}B fissions to generate a Li-7 nucleus and energetic alpha particles, which are highly destructive, with a relatively short path (10-14 mm). The specific localization of boron in rapidly dividing cells (tumor cells) is useful for destroying these cells by using Boron Neutron Capture Therapy (BNCT), without affecting normal cells. BNCT requires about 5 ppm boron-10. Therefore, BPi, which is transported to the rapidly dividing cells, is highly attractive as a BNCT reagent.

BPi as a synthetic building block for biologically active nucleotides - BPi can be used for the preparation of numerous biologically active borano-nucleotides, borano-dinucleotides, borano-oligonucleotides, etc. For instance, we have disclosed in WO 03/034978 that ATP-α-B analogues are potent $P2Y_1$-R (ATP receptor) agonists and can be utilized as therapeutic agents for the treatment of Type II diabetes. We also prepared borano-dinucleotides which are potential agonists for the $P2Y_{2/4}$-R.

In addition, these compounds may serve as inhibitors of ATP-utilizing enzymes (e. g. NTPDase) that are involved in various health disorders.

Furthermore, BPi may be used for the preparation of antisense agents targeting specific mRNA sequences.

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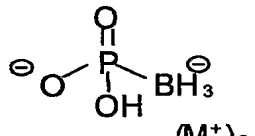
CLAIMS:

1. An inorganic boranophosphate salt of the general formula **2**:



2

wherein M is a counterion.

2. An inorganic boranophosphate salt according to claim 1 wherein the counterion M is ammonium or it is an inorganic cation derived from an alkali, alkaline earth or transition metal such as but not limited to NH_4^+, Na^+, K^+, Li^+, Ca^{++}, Mg^{++}, Ni^{++}, Cu^{++}, Fe^{++}, Fe^{+++}, Co^{++}, Zn^{++}, Pd^{++}, and Ag^+.

3. An inorganic boranophosphate salt according to claim 1 wherein the counterion M is an organic cation derived from an amine of the formula R_3NH^+, wherein R is C1-C18, preferably C1-C6, alkyl, more preferably ethyl, propyl or butyl, or two of the Rs together with the nitrogen atom to which they are attached form a 3-7 membered ring optionally containing a further heteroatom selected from the group consisting of N, S and O, such as for example pyrrolydine, piperidine, morpholine, or R is phenyl or heteroaryl such as pyridyl, imidazolyl, pyrimidinyl, and the like.

4. An inorganic boranophosphate salt according to claim 1 wherein M is NH_4^+, triethylammonium $(Et_3\ NH)^+$, or tributylammonium $(Bu_3\ NH)^+$.

5. A method for the preparation of an inorganic boranophosphate salt according to claim 1, comprising reacting tris(trimethylsilyl)-phosphite with borane-dimethylsulfide

complex of the formula $BH_3.SMe_2$, and treating the formed intermediate with the suitable base MOH in water or methanol, thus obtaining the desired salt.

6. The method according to claim 5, wherein said base is methanolic ammonia or an aqueous NH_4OH solution, thus resulting in the ammonium salt, wherein M is NH_4^+.

7. The method according to claim 5, wherein said base is tributylamine, Bu_3N, in methanol, thus resulting in the tributylammonium salt, wherein M is Bu_3NH^+.

8. The method according to claim 5, comprising treating the intermediate with triethylammonium bicarbonate buffer, thus resulting in the Et_3NH^+ salt.

9. Use of the boranophosphate salts of claim 1 as fertilizers, in detergent formulations, as additive in melts for the glass industry, in boron neutron capture therapy (BNCT) of cancer, and as synthetic building blocks in the synthesis of boranonucleotides of various lengths.